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Nuclear factor IA is expressed in astrocytomas and is associated with improved survival

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Nuclear factor IA (NFIA) is a transcription factor that specifies glial cell identity and promotes astrocyte differentiation during embryonic development. Its expression and function in gliomas are not known. Here, we examined NFIA protein expression in gliomas and its association with clinical outcome in pediatric malignant astrocytomas. We analyzed expression of NFIA by immunohistochemistry in 88 existing glioma specimens from Childrens Hospital Los Angeles and the University of Southern California. Association between NFIA expression and progression-free survival (PFS) was examined in high-grade astrocytomas for which clinical data were available ($n = 23$, all children). NFIA was highly expressed in astrocytomas of all grades, but only in a minority of cells in oligodendroglial tumors. NFIA was expressed on a higher percentage of tumor cells in low-grade astrocytomas ($91 \pm 5\%$ and $77 \pm 14\%$ in World Health Organization [WHO] I and II, respectively) compared with high-grade astrocytomas ($48 \pm 18\%$ and $37 \pm 16\%$ in WHO III and IV, respectively; $P < .001$, low- vs high-grade astrocytomas). There was a significant association between NFIA expression and PFS in children with astrocytoma WHO grade III or IV (Cox regression $P = .019$; logrank trend test for NFIA tertiles $P = .0040$ and NFIA quartiles $P = .014$). The association was not consistently significant in this small series of patients after adjustment was made for WHO grade III or IV. This is the first study to demonstrate expression of NFIA protein in astrocytomas and its association with grades of astrocytoma and PFS, suggesting that NFIA may play a role in astrocytoma biology.

Keywords: astrocytoma, glioma, nuclear factor IA (NFIA), pediatric, progression-free survival

Gliomas are the most common primary central nervous system (CNS) tumors in humans, accounting for 40%–50% of all primary intracranial neoplasms.[1] Gliomas contribute significantly to morbidity and mortality in every age group, and prognosis of patients with malignant glioma remains dismal.[2–5] Traditionally gliomas, tumors of glial origin, are classified on the basis of cellular morphology and proliferation.[6] According to the World Health Organization (WHO) classification, gliomas can be subdivided into astrocytomas, oligodendrogliomas, or mixed oligoastrocytomas, based on glial sublineage.[6]

The nuclear factor I (NFI) family of site-specific DNA-binding proteins (also known as CTF or CATT box transcription factor) are critical regulators of gliogenesis in the developing CNS.[7,8] NFIA, a member of the NFI family, has been shown to be necessary and sufficient for specification of glial identity in ventricular zone progenitors in the developing murine and avian spinal cord.[7] In addition to a role in glial specification, NFI genes are critical regulators of astrocyte differentiation in brain and spinal cord in rats, likely in part through transcriptional regulation of glial fibrillary acidic protein (GFAP) expression.[8,9] Furthermore,

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deletion of *NFIA* or *NFIB* yields defects in midline glial cell populations in postnatal mouse brain.[10,11] Indeed, NFIA is also expressed in midline structures in fetal human forebrains.[12] During glial cell development, NFI genes demonstrate dynamic patterns of expression. At the onset of embryonic gliogenesis, NFI genes are expressed in all glial precursors, and as development proceeds, they are transcriptionally repressed in the oligodendrocyte lineage, whereas their expression is maintained in the astrocyte lineage.[7] Such patterns could potentially be used as a means of identifying cells that exist at different points along the axis of glial cell differentiation.[7] Moreover, the involvement of NFIA in glial development suggests that it may be altered in glial tumors or may even play a role in glioma development. Therefore, as our first step in investigating a possible role for NFIA in gliomas, we analyzed expression of NFIA in glial tumors and its association with patient survival. Our data show that higher expression of NFIA protein in astrocytomas is associated with longer survival, suggesting that NFIA may play a role in the biology of human astrocytomas.

Materials and Methods

Tissue Samples

This retrospective study was approved by the Institutional Review Boards at Childrens Hospital Los Angeles and the University Hospital at University of Southern California (USC). Tissues included were formalin-fixed brain tumor samples that were acquired between 1990 and 2007 from 120 patients at Childrens Hospital Los Angeles or the University Hospital at USC. After pathology review, 9 cases containing insufficient or poorly preserved tumor material were excluded. Also excluded from the analysis was 1 patient with a diagnosis of giant cell glioblastoma, which by some is considered to have a different behavior than glioblastoma multiforme (GBM). The remaining 110 eligible tissue samples included 88 human gliomas (74 astrocytomas, 12 oligodendrogliomas, and 2 oligoastrocytomas), 12 nonglial tumors (meningioma, schwannoma, and lymphoma, 4 of each) resected at the time of diagnosis, and 10 non-neoplastic brain tissues from epilepsy surgery or autopsies. Information (age, gender, therapy, progression-free survival [PFS], and overall survival [OS]) on patients with high-grade astrocytomas was only available for samples from Childrens Hospital Los Angeles (23 pediatric patients; Table 1, retrospective chart review). Samples from 19 of the 110 patients (3 WHO III astrocytomas, 6 WHO IV astrocytomas, and 10 oligodenroglial tumors) were from individuals over age 18 years who underwent surgery at USC, since high-grade gliomas and oligodendrogliomas are relatively less common in pediatric patients. However, clinical information was not available on them.

Table 1. Clinical parameters of patients with AA and GBM analyzed in Fig. 5

Diagnosis	Age (y)	Sex	NFIA (%)	PFS** (y)	OS** (y)	Status	Surgery	RT	Chemo
AA (WHO III)	7.9	M	77	0.25	0.86	DOD	P	Y	Y
AA	4.1	M	74	1.94	3.72+	AWD	GT	Y	Y
AA	9.6	F	70	0.26+	0.26	*DUC	GT	N	Y
AA	4.9	F	60	2.40	2.41+	AWD	P	Y	Y
AA	4.9	M	54	0.15+	0.15+	AWD	P	Y	Y
AA	4.7	M	54	1.83+	1.83+	AWD	GT	Y	Y
AA	3.2	F	52	0.58	8.67+	AWD	P	Y	Y
AA	14.1	F	51	1.41	1.42	DOD	P	Y	Y
AA	8.7	M	50	0.51	0.60	DOD	P	Y	Y
AA	11.5	F	50	0.75	0.84	DOD	P	Y	Y
AA	12.1	M	49	0.61	1.23	DOD	GT	Y	Y
AA	4.8	M	48	0.17+	0.17+	AWD	P	N	Y
AA	1.7	M	40	1.35	9.31+	AWD	GT	Y	Y
AA	2.4	F	40	11.14+	11.14+	NED	P	Y	Y
AA	11.2	F	33	0.64	0.89	DOD	P	Y	Y
AA	15.1	M	30	0.51	0.71	DOD	GT	Y	Y
AA	1.7	F	13	0.26	6.20+	AWD	GT	Y	Y
GBM (WHO IV)	6.9	F	40	1.09	5.53+	AWD	GT	Y	Y
GBM	18.7	F	31	0.60	1.32	DOD	GT	Y	Y
GBM	11.3	F	28	0.45	3.06	DOD	GT	Y	Y
GBM	14.6	M	25	0.11	0.28	DOD	GT	Y	Y
GBM	19.8	F	23	0.12	0.99+	AWD	GT	Y	Y
GBM	5.2	M	22	0.26	0.79	DOD	B	Y	Y

Abbreviations: DOD, dead of disease; GT, gross total resection; *DUC, dead of unrelated causes (pneumonia, in this child); P, partial resection; AWD, alive with disease; B, biopsy; NED, no evidence of disease; ** +, censored in survival analysis.

Immunohistochemistry

To examine expression of NFIA, immunohistochemical staining with DAB detection on paraffin-embedded tumor-containing sections was performed using the DAKO Envision + system (DakoCytomation, Carpinteria, California, K4009). Double-labeled immunohistochemistry for NFIA and GFAP was performed using DAKO Doublestain Envision Kit (DakoCytomation, K1395) according to the manufacturer's specifications. After deparaffinization, heat-based antigen retrieval (10 mM citrate, 99°C, 30 minutes) in a steamer was performed on all sections to enhance immunodetection. Non-specific antibody binding was blocked by incubation with a protein-blocking reagent (DAKO) for 20 minutes prior to incubating with a primary antibody. The following antibodies were used: NFIA 1:3000 (rabbit polyclonal, Geneka, now Active Motif®) and GFAP 1:50 (mouse monoclonal-IgG1, DAKO). The NFIA rabbit polyclonal antibody is directed against amino acids 478–492 of human NFIA.[13] Specificity of this NFIA antibody (Western blot, immunoabsorption, and knockout tissue analysis) has been demonstrated by Plachez et al.[13] and by the manufacturer (http://www.activemotif.com/catalog/details/39036.html). Stains replacing NFIA primary antibody with rabbit IgG or lacking the primary antibody were used as negative controls for the NFIA immunohistochemical stains (both gave a negative stain). Hematoxylin and eosin (H&E) staining was performed according to standard procedures.

Scoring of Immunohistochemically Stained Sections

An independent neuropathologist (I.G.-G.) who was blinded to the originally assigned pathological diagnosis and clinical outcome reconfirmed the neuropathologic diagnosis on newly cut paraffin-sections, using the guidelines of the WHO.[6] Quantification of NFIA expression was performed by 2 independent investigators (I.G.-G. and H.-R.S.) who were blinded to each other's analysis, to the diagnosis, and to the WHO grade assigned to the sample. NFIA expression was quantified by counting the number of tumor cells that expressed NFIA as a percentage of the total number of tumor cells counted within 3 HPFs (high-power fields; magnification ×400; at least 200 tumor cells per 3 HPFs). Cells with staining of NFIA were interpreted as NFIA-expressing cells. Endothelial cells, which lack NFIA immunoreactivity, were used as an internal negative control. Other negative controls included isotype control antibody in place of the primary antibody and omission of the primary antibody. The value used for statistical analysis was the average of the readings by the 2 counting investigators for each tumor. The inter-investigator variation in percent cells expressing NFIA was less than 10%.

Statistical Analyses

Survival analysis methods, specifically the product limit (Kaplan–Meier) estimate, the logrank test, and Cox regression analysis,[14] were used to evaluate the association of NFIA expression with PFS and OS. PFS was defined as the minimum interval from the date of diagnosis to the date of tumor recurrence, progression, or the last follow-up. OS was defined as the interval from the date of diagnosis to death from any cause or the last follow-up. Several different analyses of the association of PFS with NFIA expression were performed—Cox regression on continuous NFIA; logrank trend test with NFIA grouped by tertiles and quartiles (ie, 3 or 4 nearly equal-sized groups)—to examine the consistency of the result. One-way analysis of variance (ANOVA) was used to test the difference in percentage of cells expressing NFIA by tumor grade.[15] Statistical computation was performed using Stata 9.2[16] and Prism GraphPad 5.0 for MacIntosh (GraphPad Software, San Diego, California). Query of the Cancer Genome's Atlas Repository of Molecular Brain Neoplasia Data (REMBRANDT; http://caintegrator-info.nci.nih.gov/rembrandt) was performed on December 3, 2008, according to the instructions on the website, using NFIA as the search term.

Results

NFIA is Expressed in Human Astrocytes

NFIA is a transcription factor that determines astroglial cell fate during early development and is expressed in cells of astrocytic lineage in developing murine and avian embryos.[7] To determine whether NFIA was also expressed in normal human astrocytes, we analyzed normal non-neoplastic brains by immunohistochemistry ($n = 10$; Fig. 1). Indeed, NFIA was expressed in approximately 10%–20% of cells in the lower layers (layers 5–6) of normal human cerebral cortex and in white matter (Fig. 1A and C). Expression was exclusively nuclear. Interestingly, NFIA expression was higher in the subventricular zone (SVZ; ~30%–40% of cells; Fig. 1B). Histologically, NFIA-stained cells showed features of astrocytes, with larger nuclei and abundant cytoplasm compared with oligodendrocytes. Double immunolabeling with NFIA and GFAP (marker for differentiated astrocytes) demonstrated that NFIA was predominantly (80%–90%) expressed in GFAP-positive cells in the normal cerebral cortex (arrows, Fig. 1D). In the cerebral cortex, NFIA was not expressed in neurons (GFAP-negative large cells with central nucleus; arrowheads, Fig. 1D). These data indicate that in normal human cerebral cortex, NFIA is predominantly expressed in cells of astrocytic lineage.

NFIA is Differentially Expressed in Human Astrocytomas

Given that NFIA marks cells of astrocytic lineage, we next examined expression of NFIA in human astrocytomas ($n = 74$) using immunohistochemistry, as described in Materials and Methods. Figure 2 shows that NFIA was expressed in nuclei of tumor cells in all grades of astrocytoma. Interestingly, we found an inverse

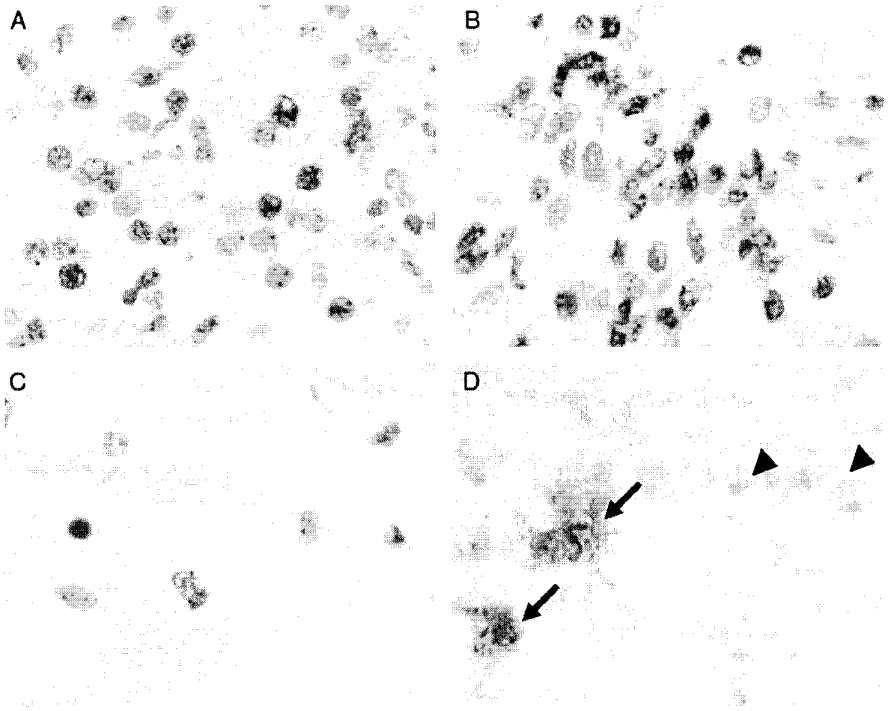


Fig. 1. NFIA expression is restricted to astrocyte-lineage cells in normal human cerebral cortex. Imunohistochemical analysis of NFIA (brown) in paraffin sections of human gliomas, counterstained with hematoxylin (blue) as described in the Materials and Methods section. (A) Cerebral cortex, (B) SVZ, (C) white matter, and (D) double staining with NFIA (brown) and GFAP (pink). Arrows show astrocytic cells, which express both NFIA and GFAP. Arrowheads indicate neurons, which express neither marker.

association between the percentage of tumor cells expressing NFIA and the WHO grade of the astrocytomas, with higher proportion of NFIA-expressing tumor cells in the lower-grade astrocytomas ($P < .001$, low-grade astrocytomas vs high-grade astrocytomas, Fig. 2 and Table 2). In both pilocytic astrocytoma (WHO grade I) and astrocytoma (WHO grade II), we found cells expressing NFIA uniformly throughout the tumor (Fig. 2A), with all pilocytic tumors uniformly expressing NFIA in over 80% of the tumor cells. The other astrocytoma grades had greater heterogeneity of NFIA expression among tumors within the same grade (Fig. 2B). Of the 12 GBMs we analyzed, 2 demonstrated perivascular invasion of tumor cells around the tumor border. Interestingly, the perivascularly infiltrating tumor cells expressed NFIA at an exceptionally large proportion compared with tumor cells within the main tumor mass (Fig. 3). This expression pattern suggests that NFIA may play a yet-unknown role in astrocytoma biology, and possibly in invasion.

NFIA is Expressed Only Minimally in Oligodendrogliomas

During embryonic development, NFIA is expressed mostly in cells of astrocyte lineage, but less so in cells of oligodendrocyte lineage.[7] Therefore, we next compared NFIA expression in the astrocytomas with expression in nonastrocytic gliomas: 12 oligodendroglial tumors and 2 oligoastrocytomas (mixed glial tumors; Fig. 4). In the oligoastrocytomas we examined (immunohistochemistry), only cells with astrocytic features (increased pleomorphism and atypia; arrows) expressed NFIA, whereas cells with oligodendroglial features (round nuclei and perinuclear halos; arrowheads) did not express NF1A (Fig. 4, top panel). Quantification of NFIA-expressing cells in the oligodendrogliomas revealed that a mean of only $4.0 \pm 3\%$ of all tumor cells expressed NFIA (Figs 2B and 4 middle and bottom panels). This expression was significantly lower than the expression of NFIA we found in astrocytomas ($P < .001$). Similarly, in the nonglial brain tumors we examined (schwannomas, lymphomas, and meningiomas, $n = 4$ tumors for each type), NFIA was also expressed in less than 3% of the tumor cells (Table 2 and Supplementary Material, Fig. S1). Taken together, these data demonstrate that although NFIA was highly expressed in astrocytomas, it was minimally expressed in oligodendrogliomas and in nonglial brain tumors.

Expression of NFIA is Associated with PFS in Malignant Astrocytomas

Our immunohistochemistry data show that in malignant astrocytomas (anaplastic astrocytoma and GBM, WHO III and IV, respectively), NFIA was expressed in fewer of

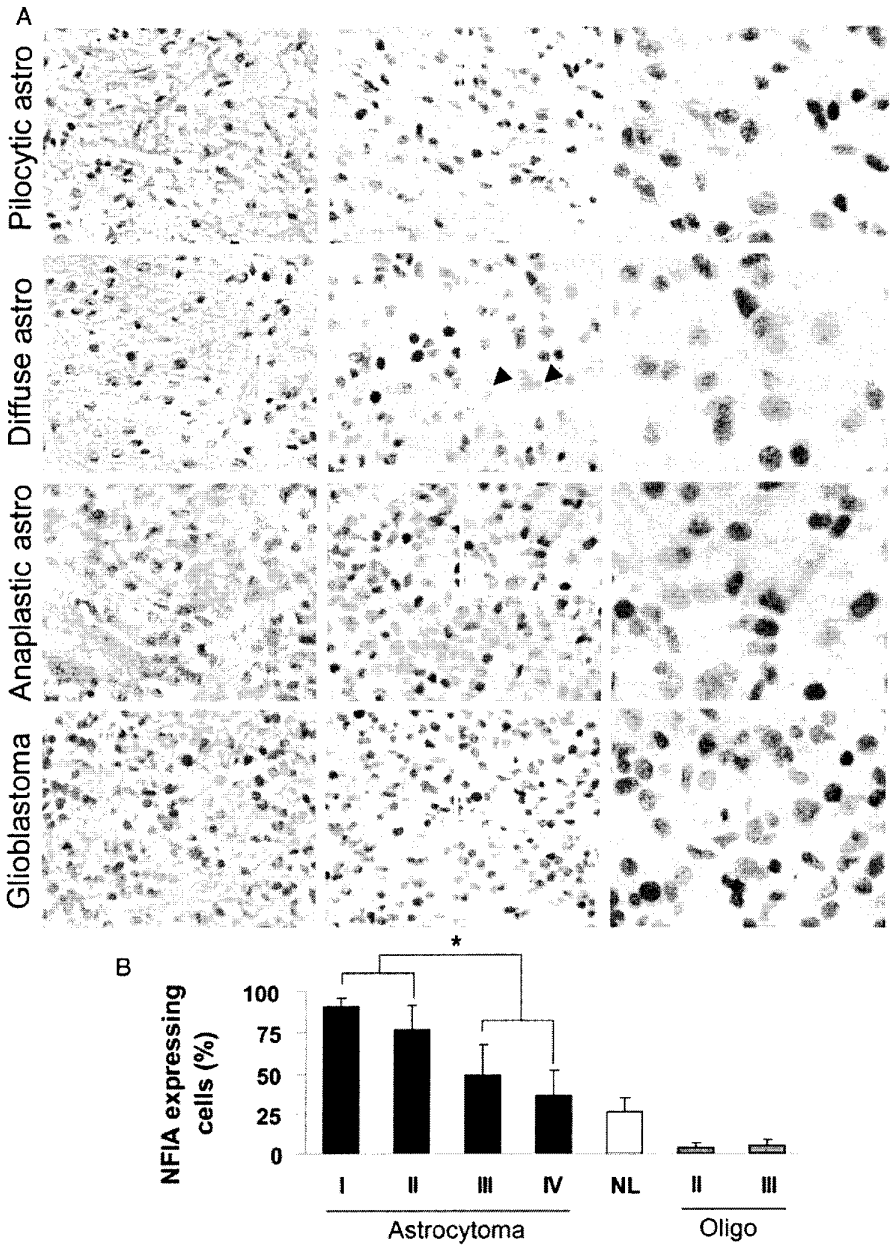


Fig. 2. NFIA is highly expressed in human astrocytomas. (A) H&E and immunohistochemistry for NFIA of 4 representative pediatric astrocytomas WHO grades I–IV. Left column is H&E stain (×400), middle column is anti-NFIA stain (×400), and right column is a 2-fold magnification of the inset from the middle column. Endothelial cells, which lack NFIA immunoreactivity, were used as an internal negative control (arrowheads). (B) Quantification of NFIA expression in pediatric and adult gliomas and normal brains. Bars represent mean ± *SD* for pilocytic astrocytoma (I, $n = 21$), astrocytoma (II, $n = 21$), anaplastic astrocytoma (III, $n = 20$), and GBM (IV, $n = 12$). Comparison is with nontumor containing cerebrum (NL, $n = 10$), oligodendroglioma (II, $n = 7$), and anaplastic oligodendroglioma (III, $n = 5$). *$P < .0001$ in the continuum of the 4 WHO grades of astrocytoma (one-way ANOVA) and $P < .001$ between the low-grade (WHO I and II) and the high-grade (WHO III and IV) astrocytomas (Student's *t*-test). *P* value is also <.0001 between astrocytomas and oligodendrogliomas (*t*-test). See Table 2 for details.

the tumor cells compared within low-grade astrocytomas (WHO grade I and II), where it was highly expressed (Fig. 2B). This suggested that higher NFIA expression may be associated with better survival. We therefore asked whether NFIA expression was also inversely associated with outcome among the malignant

Table 2. NFIA is preferentially expressed in astrocytomas (IHC) and is higher in low-grade astrocytoma compared with high-grade astrocytoma

	% of tumor cells expressing NFIA, mean ± *SD* (*n*)
Astrocytoma	
WHO I	91 ± 5 (21)
WHO II	77 ± 14 (21)
WHO III	48 ± 18 (20)
WHO IV	37 ± 16 (12)
Oligodendroglioma	
WHO II	3 ± 3 (7)
WHO III	5 ± 3 (5)
Nonglial tumors	
Schwannoma	1 ± 0.8 (4)
Meningioma	2 ± 0.7 (4)
Lymphoma	0 (4)
Normal human brain	26 ± 8

P values (Student's *t*-test) are: $P = .0003$ between WHO I and II astrocytoma, $P < .0001$ between WHO II and III astrocytoma, $P = .0695$ between WHO III and IV astrocytoma, and $P < .0001$ between WHO I + II and III + IV. $P < .0001$ between all astrocytomas and normal brain, nonglial brain tumors, or oligodendrogliomas.

WHO III and IV astrocytomas, both of which carry a poor prognosis.

We first analyzed the publicly available REMBRANDT[17] of the Cancer Genome Atlas, which contains microarray expression and OS data for adult patients with GBM. Data are available for NFIA expression using 4 probesets and are compared with nontumor brain samples. Analyzing these data, we found that in 2 of the 4 NFIA probesets, patients whose GBM (WHO grade IV astrocytoma) had a high NFIA mRNA level (defined as ≥2-fold upregulated compared with non-tumor samples) benefited from significantly longer OS ($P < .05$) compared with patients whose GBM NFIA level was intermediate (defined as expression level between 2-fold upregulated and 2-fold downregulated compared with nontumor samples; probesets 224976_at and 224970_at; Fig. 5). Only 1 of the 4 probesets (1557639_at) had sufficient number of GBM patients with low NFIA expression level (defined as NFIA dowregulated ≥2-fold compared with nontumor tissues; $n = 16$ patients with downregulated NFIA). This probeset showed that low NFIA expression was significantly associated with worse OS when compared with intermediate NFIA- or high NFIA-expressing GBMs (Fig. 5). One probeset (224975_at) did not show a significant difference in

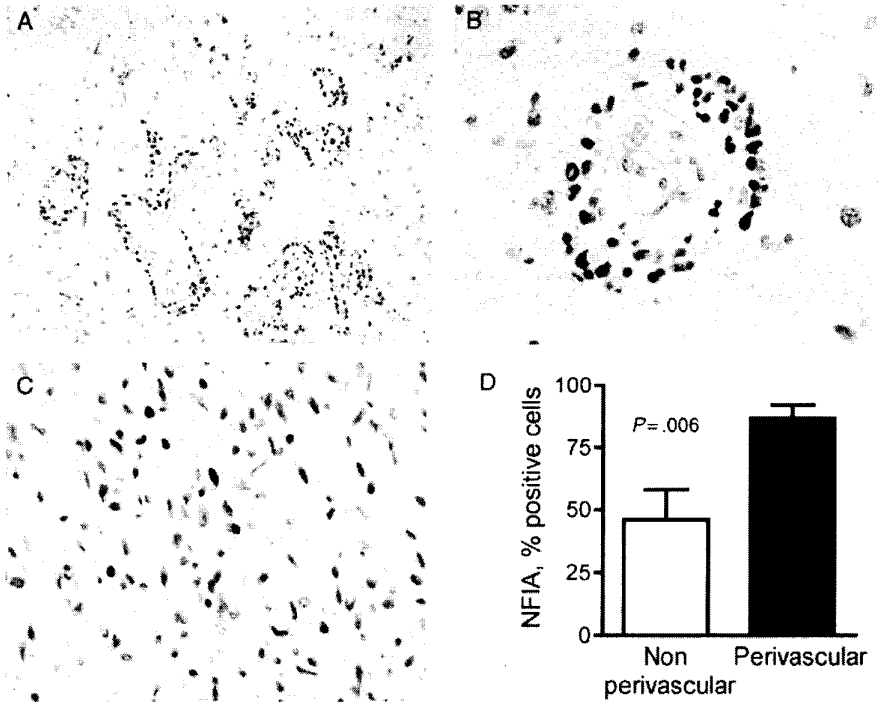


Fig. 3. NFIA is expressed highly in perivascular tumor cells in glioblastomas. Immunohistochemical stain for NFIA in GBM: (A and B) perivascular infiltrating tumor cells at the tumor border (top row; magnification: left panel ×100 and right panel ×400). (C) Nonperivascular area of tumor showing lower expression of NFIA in the tumor cells (same patient; magnification ×400). (D) Percent NFIA positive cells compared with total tumor cells in perivascular compared with nonperivascular areas in 2 cases of GBM. Shown is the analysis of 3 representative high-power fields from 2 invasive adult GBMs that were quantified for perivascular NFIA expressing tumor cells compared with nonperivascular ones. NFIA-positive cells are brown, nuclear stain is blue.

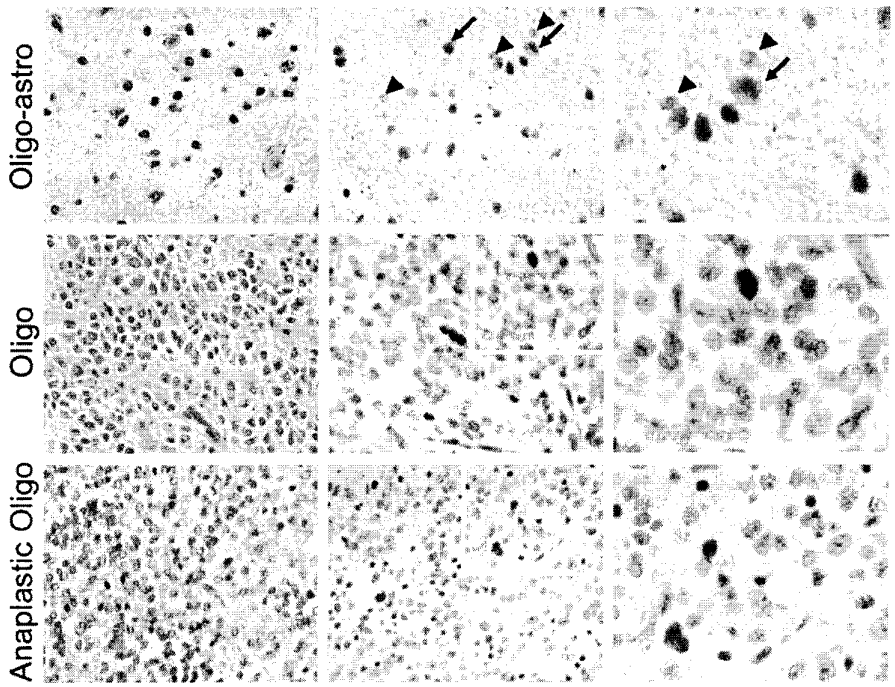


Fig. 4. NFIA is only minimally expressed in oligodendroglial tumors. Immunohistochemical analysis of NFIA in oligoastrocytomas (top row), oligodendrogliomas (middle row), and anaplastic oligodendrogliomas (lower row) was performed as in Fig. 2. Shown are representative fields. Quantification of the percent NFIA-positive tumor cells is shown in Fig. 2B. In oligoastrocytomas, cells with astrocytic features (increased pleomorphism and atypia; arrows) expressed NFIA, but cells with oligodendroglial features (round nuclei and perinuclear halos; arrowheads) were negative. In the pure oligodendrogliomas (middle and lower panels), only rare cells expressed NFIA.

survival between high NFIA and intermediate NFIA-expressing tumors ($P = .0701$). Nonetheless, this probeset showed a trend similar to the findings seen with the other probesets (Fig. 5). This suggests that in adult patients with GBM, higher NFIA expression in the tumor was associated with improved OS.

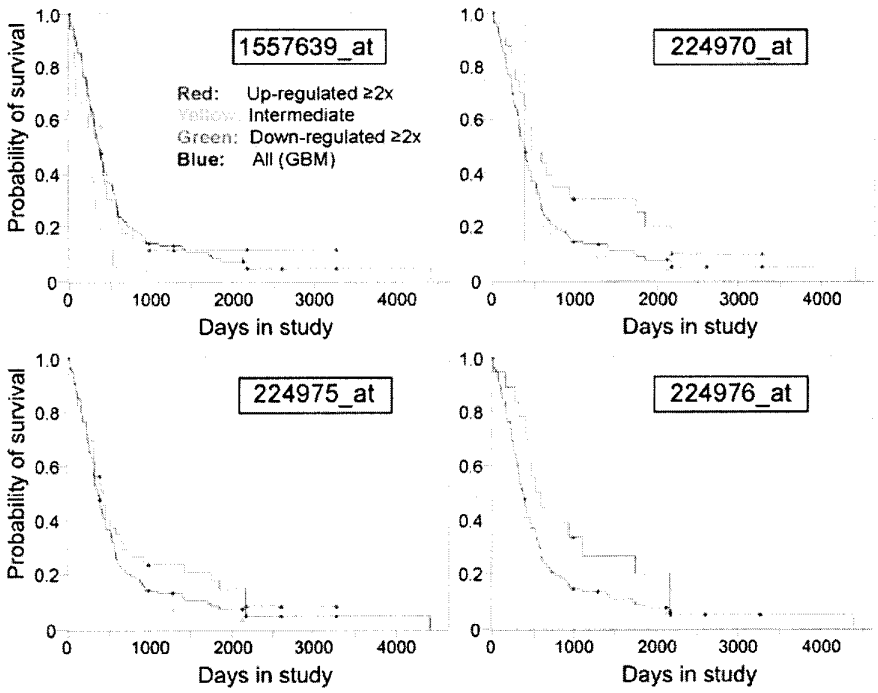
These findings prompted us to examine PFS and OS of the patients with WHO III and IV astrocytomas whose tumors were analyzed in Fig. 2 and whose clinical information was available ($n = 23$, all pediatric patients at Childrens Hospital Los Angeles). In these 23 pediatric patients, PFS was significantly associated with NFIA expression in univariate Cox regression ($P = .019$, continuous NFIA). When dividing NFIA expression into either 3 or 4 groups that are as equal as possible, PFS was also significantly associated with NFIA expression (tertiles: $P = .0040$, quartiles: $P = .014$, logrank test for trend, Fig. 6). PFS association with NFIA was not significant if the group was divided at the median ($P > .10$), but 2-group comparisons of continuous variables are known to be inefficient.[18] The association also was not consistently significant in these analyses when adjustment was made for WHO grade III vs IV, although all of these analyses nominally showed lower failure risk with higher NFIA expression. Figure 6 and Table 3 show the difference in PFS and OS among these 23 pediatric patients. There was no significant association between NFIA expression and OS in this small series ($P > .10$, logrank test for trend), in which 1 patient died of pneumonia without evidence of disease 3 months from diagnosis and another was lost to follow-up less than 2 months after surgery. Interestingly, in the much larger REMBRADNT adult GBM mRNA microarray data set presented above (Fig. 5), higher NFIA was associated with longer OS (Fig. 5), corroborating the PFS data calculated based on NFIA protein expression by IHC in the pediatric high-grade astrocytoma patients.

Taken together, our data show that NFIA expression was higher in WHO grade I and II astrocytomas compared with WHO grade III and IV astrocytomas and that in the WHO grade III and IV astrocytomas a higher expression of NFIA was associated with better PFS. Interestingly, NFIA expression was only minimal in oligodendrogliomas compared with the astrocytomas.

Discussion

The goal of our study was to assess expression of the glial fate determinant NFIA in human gliomas compared with normal brain and nonglial brain tumors. We also examined whether NFIA expression was associated with survival.

Our data show that NFIA was differentially expressed in astrocytomas: it was highly expressed in the low-grade astrocytomas (WHO I and II) compared



Probeset:	1557639_at	224970_at	224975_at	224976_at
Number of Patients (*n*):				
Upregulated ≥2x	33	23	39	18
	56	81	66	87
Downregulated ≥2x (i.e.≤0.5x of normal)	16	1	–	–

	1557639_at	224970_at	224975_at	224976_at
P value (logrank):				
Upregulated vs intermediate	0.4621	**0.0199**	0.0701	**0.0325**
Upregulated vs Downregulated	0.0608	0.4105	NA	NA
Downregulated vs Intermediate	**0.0013**	0.836	NA	NA
Upregulated vs all others	0.9199	**0.019**	NA	NA
Downregulated vs all others	**0.0032**	0.736	NA	NA
Intermediate vs all others	0.0764	**0.0236**	NA	NA

Fig. 5. Upregulation of NFIA mRNA (microarrays) is associated with longer OS in adult GBM patients. Kaplan–Meier plots (OS) analyzed and derived from the publicly available REMBRANDT expression microarray glioma data set for adult GBM patients grouped according to NFIA mRNA expression level (4 of 4 NFIA probesets available).[17] Red curves (high NFIA expression): ≥2-fold increase in NFIA expression compared with nontumor brain samples, yellow curves (intermediate): intermediate between >2-fold increase and >2-fold decrease of NFIA mRNA (ie, >0.5- and <2.0-fold NFIA expression level compared with nontumor brain sample), or green (low): ≥2-fold decrease in NFIA expression compared with nontumor tissue. Blue line depicts all GBM samples combined. The table under the 4 panels shows sample number and *P* values calculated by the REMBRANDT website.

with the high-grade astrocytomas (WHO III and IV). To date, the only other analysis of NFI genes in gliomas has been in a recent microarray analysis validated by real-time PCR of 20 primary GBMs that found overexpression of NFIA mRNA compared with that of normal brain.[19] However, our data provide the only analysis to date to examine the association of NFIA with survival in astrocytomas or in any other cancer. Interestingly, a link of NFIA to human leukemia was recently reported, with the finding of NFIA mutations of yet-unknown significance in 2 patients with chronic malignant myeloid disease.[20]

Although our data suggest involvement of NFIA in astrocytoma biology, the function of NFIA in gliomas is not clear at this time. Since NFIA is required for glial differentiation in embryonic development,[7,10] it is

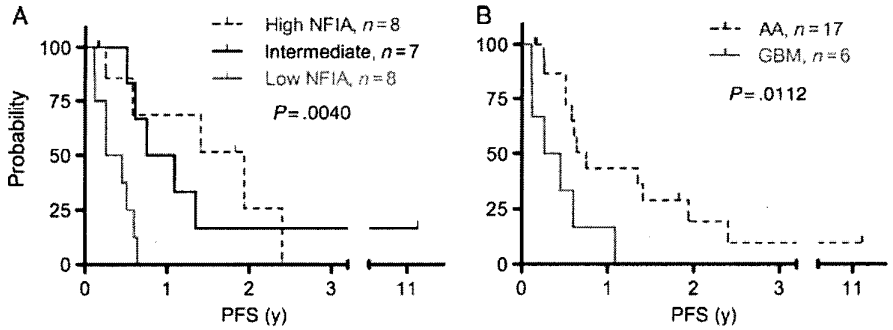


Fig. 6. Higher NFIA expression is associated with longer PFS in pediatric high-grade astrocytoma patients (WHO III and IV). Kaplan–Meier plots of PFS in pediatric patients with high-grade astrocytoma (WHO III and IV). (A) Patients grouped into 3 equal groups according to % tumor cells expressing NFIA: high NFIA (blue dashed), intermediate NFIA (black, solid), and low NFIA (red, solid). $P = .0040$ was by logrank test for trend. Tick marks indicate length of follow-up in 4 patients without disease progression. (B) Patients grouped by WHO grade: grade IV, GBM (red line), and grade III, anaplastic astrocytoma (dashed blue line). $P = .0112$ by logrank test (Mantel–Cox). Numbers of patients are shown in the panels. Median survival is shown in Table 3.

Table 3. Median PFS is longer in pediatric high-grade astrocytoma patients with higher NFIA expression

	AA	GBM	*P* value	% cells with NFIA expression			*P* value
				Low	Intermediate	High	
Median PFS (d)	274	95	.0112	95	274	709	.0040
Median OS (d)	518	482	.62	325	449	Not reached	.217
n	17	6		8	7	8	

P values between tertiles are calculated by logrank test for trend. *P* values between AA (WHO III) and GBM (WHO IV) are calculated by logrank test.

possible that its higher expression in the more differentiated astrocytomas (WHO I and II) may reflect its role in promoting astrocyte differentiation, providing a possible tumor-suppressive role. On the other hand, the requirement of NFIA for maintenance of the glial progenitor cell pool in embryonic spinal cord[7] may hint at a possible contribution to tumor initiating cells. This suggests a complex mechanism of action that may depend on the genetic and cellular context in which NFIA is expressed, as well as a specific use of one or more of the isoform(s) of NFIA.[8,21–23] Interestingly, in chicken embryo fibroblasts, NFIA was not oncogenic and its expression even rendered the cells resistant to transformation by nuclear oncogenes, such as *jun*, *fos*, and *myc* (all encoding transcription factors),[24] again suggesting a tumor-resisting function. However, these NFIA expressing chicken cells were readily transformed by cytoplasmic oncogenes, such as *src*, *raf*, *ras*, and *fms*,[24] indicating retention of a tumor-permissive role for certain signaling pathways. If a dual role is found for NFIA in astrocytomas, this may not be surprising in view of its many isoforms and the complexity of function already known in other transcription factors such as WT1, which originally was discovered as a tumor-suppressor gene, but in another isoform, was later found to have oncogenic function.[25] Alternatively, genetic context may also play a role in determining possible oncogenic/permissive vs tumor-suppressive function of NFIA as demonstrated in certain oncogenes, such as *myc* and *ras*, which induce apoptosis in the presence of p53 while promoting transformation in the absence of p53.[26–28] It is interesting to note that NFI can bind to an element in the p53 promoter that contributes to the basal activity of the promoter function of p53 in a tissue-specific and mutually exclusive manner with YY1, a zinc finger transcription factor that can promote (eg, *c-myc*) or repress (eg, *c-fos*) other promoters.[29]

Our observation that NFIA protein is highly enriched in perivascular tumor cells in the invasive tumor border compared with nonperivascular tumor cells within the main GBM mass suggests that in high-grade astrocytomas, NFIA may function (either positively or negatively) in tumor cell invasion and/or migration. Consistent with a role of NFIA in migration, the adhesion molecules ephrin B1 and N-cadherin were recently found to be targets of NFIA in cerebellar granular neurons.[30]

Identification of markers that distinguish astrocytomas from oligodendroglial tumors has long been a goal of neuropathologists and neuro-oncologists. However, markers, such as Olig2 and STAT3, which are thought to function in glioma formation, are similarly expressed in both astrocytomas and oligodendrogliomas, precluding their diagnostic use.[31,32] Therefore, our observation that NFIA was expressed in all grades of astrocytomas, but only minimally in oligodendrogliomas is interesting,

and suggests that future studies may find NFIA to be a useful diagnostic marker to distinguish between these 2 glial tumors. Supporting this, preliminary examination of a small number of oligodendroglial tumors showed that cellular expression of NFIA and the oligodendroglial marker, Olig2 were mutually exclusive (data not shown, unpublished data). These data are consistent with developmental expression patterns, where NFIA is preferentially expressed in astrocytes but not in oligodendrocytes. Interestingly, during some stages of spinal cord development, Olig2 antagonizes the ability of NFIA to promote astrocyte differentiation, and the suppression of Olig2 by NFIA seems to depend on the relative levels of the 2 proteins.[7] Additionally, Olig2 can transcriptionally repress NFIA expression. In the *Olig2* $-/-$ mouse, there is a derepression of NFIA expression, suggesting that in oligodendrogliomas Olig2 may transcriptionally repress NFIA expression (B.D., unpublished data). A more comprehensive study is needed to determine whether NFIA may be useful as a diagnostic astrocytic marker to differentiate astrocytic tumors from oligodendroglial tumors in cases that are difficult to assess based on morphology alone.

Our demonstration that NFIA protein expression in astrocytomas is associated with longer PFS was significant despite the relatively small group of patients available for analysis of PFS ($n = 23$ children). Larger-scale studies, such as those possible in adult patients, will be needed in order to determine whether NFIA may be a potential prognostic marker in high-grade astrocytomas. Our immunohistochemical results are supported by the analysis of the publicly available REMBRANDT microarray data set (Fig. 5), which showed that OS was longer in adult patients whose GBM tumors expressed $\geq$2-fold higher NFIA mRNA (relative to NFIA in nontumor samples), when compared with GBM patients with intermediate or low NFIA. Scrideli et al.[19] showed by quantitative real-time PCR that expression of NFIA mRNA in 20 GBMs was more than 13 times higher than in 10 samples of non-neoplastic white matter, but did not examine association with PFS or OS. Our immunohistochemistry results showed that the fraction of cells expressing NFIA in GBM was higher than in the normal brain (Fig. 2B, $P < .001$) corroborating the data of Scrideli et al.[19] at the protein level. The findings of Schuur et al.[24] that NFIA expression in chick embryo fibroblasts renders them resistant to transformation by nuclear oncogenes may hint at a mechanism that may be related to our findings of association of higher NFIA with improved outcome in the high-grade astrocytomas and its higher expression in the low-grade astrocytomas. The protection from transformation by NFIA in the chick embryo fibroblasts may suggest that retention of NFIA in astrocytoma may protect cells from further transforming events, whereas its loss (or perhaps its cytoplasmic localization) may expose the cells to increased susceptibility to further malignant transformation.[24]

In summary, our data demonstrate differential expression of NFIA in low- vs high-grade astrocytomas and association between NFIA protein expression and improved PFS in children with high-grade astrocytomas. This is supported by the analysis of the REMBRANDT GBM microarray database. These findings, combined with current knowledge, suggest that NFIA may have an important role in astrocytoma biology.

Supplementary Material

Supplementary material is available at *Neuro-Oncology* online.

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References

1. CBTRUS. 2007–2008, Central Brain Tumor Registry of the United States.
2. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* 2005;352(10):997–1003.
3. Keles GE, Chang EF, Lamborn KR, et al. Volumetric extent of resection and residual contrast enhancement on initial surgery as predictors of outcome in adult patients with hemispheric anaplastic astrocytoma. *J Neurosurg.* 2006;105(1):34–40.
4. Sanai N, Berger MS. Glioma extent of resection and its impact on patient outcome. *Neurosurgery.* 2008;62(4):753–764; discussion 264–266.
5. Sposto R, Ertel IJ, Jenkin RD, et al. The effectiveness of chemotherapy for treatment of high grade astrocytoma in children: results of a randomized trial. A report from the Childrens Cancer Study Group. *J Neurooncol.* 1989;7(2):165–177.
6. Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* 2007;114(2):97–109.
7. Deneen B, Ho R, Lukaszewicz A, et al. The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron.* 2006;52(6):953–968.

8. Gronostajski RM. Roles of the NFI/CTF gene family in transcription and development. *Gene*. 2000;249(1–2):31–45.
9. Cebolla B, Vallejo M. Nuclear factor-I regulates glial fibrillary acidic protein gene expression in astrocytes differentiated from cortical precursor cells. *J Neurochem*. 2006;97(4):1057–1070.
10. das Neves L, Duchala CS, Tolentino-Silva F, et al. Disruption of the murine nuclear factor I-A gene (*Nfia*) results in perinatal lethality, hydrocephalus, and agenesis of the corpus callosum. *Proc Natl Acad Sci USA*. 1999;96(21):11946–11951.
11. Steele-Perkins G, Plachez C, Butz KG, et al. The transcription factor gene *Nfib* is essential for both lung maturation and brain development. *Mol Cell Biol*. 2005;25(2):685–698.
12. Ren T, Anderson A, Shen WB, et al. Imaging, anatomical, and molecular analysis of callosal formation in the developing human fetal brain. *Anat Rec A Discov Mol Cell Evol Biol*. 2006;288(2):191–204.
13. Plachez C, Lindwall C, Sunn N, et al. Nuclear factor I gene expression in the developing forebrain. *J Comp Neurol*. 2008;508(3):385–401.
14. Cox DR, Oakes D. *Analysis of Survival Data*. New York: Chapman and Hall; 1984.
15. Kirk RE. Experimental Design: Procedure for the Behavioral Sciences. Pacific Grove, CA: Brooks/Cole; 1995.
16. Stata C. Stata Statistical Software: Release 9, StataCorp LP. TX: College Station; 2005.
17. REMBRANDT. National Cancer Institute. http://caintegrator-info.nci.nih.gov/rembrandt, 2005.
18. Cox DR. Note on grouping. *J Am Stat Assoc*. 1957;52:534–537.
19. Scrideli CA, Carlotti CG Jr, Okamoto OK, et al. Gene expression profile analysis of primary glioblastomas and non-neoplastic brain tissue: identification of potential target genes by oligonucleotide microarray and real-time quantitative PCR. *J Neurooncol*. 2008;88(3):281–291.
20. Bernard F, Gelsi-Boyer V, Murati A, et al. Alterations of NFIA in chronic malignant myeloid diseases. *Leukemia*. 2009;23(3):583–585.
21. Baumeister H, Gronostajski RM, Lyons GE, et al. Identification of NFI-binding sites and cloning of NFI-cDNAs suggest a regulatory role for NFI transcription factors in olfactory neuron gene expression. *Brain Res Mol Brain Res*. 1999;72(1):65–79.
22. Ling G, Hauer CR, Gronostajski RM, et al. Transcriptional regulation of rat CYP2A3 by nuclear factor 1: identification of a novel NFI-A isoform, and evidence for tissue-selective interaction of NFI with the CYP2A3 promoter in vivo. *J Biol Chem*. 2004;279(27):27888–27895.
23. Xie Y, Madelian V, Zhang J, et al. Activation of the NPTA element of the CYP2A3 gene by NFI-A2, a nasal mucosa-selective nuclear factor 1 isoform. *Biochem Biophys Res Commun*. 2001;289(5):1225–1228.
24. Schuur ER, Kruse U, Iacovoni JS, et al. Nuclear factor I interferes with transformation induced by nuclear oncogenes. *Cell Growth Differ*. 1995;6(3):219–227.
25. Hohenstein P, Hastie ND. The many facets of the Wilms' tumour gene, WT1. *Hum Mol Genet*. 2006;15(Spec 2):R196–R201.
26. Serrano M, Lin AW, McCurrach ME, et al. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*. 1997;88(5):593–602.
27. Hermeking H, Eick D. Mediation of c-Myc-induced apoptosis by p53. *Science*. 1994;265(5181):2091–2093.
28. Henriksson M, Selivanova G, Lindström M, et al. Inactivation of Myc-induced p53-dependent apoptosis in human tumors. *Apoptosis*. 2001;6(1–2):133–137.
29. Furlong EE, Rein T, Martin F. YY1 and NF1 both activate the human p53 promoter by alternatively binding to a composite element, and YY1 and E1A cooperate to amplify p53 promoter activity. *Mol Cell Biol*. 1996;16(10):5933–5945.
30. Wang W, Mullikin-Kilpatrick D, Crandall JE, et al. Nuclear factor I coordinates multiple phases of cerebellar granule cell development via regulation of cell adhesion molecules. *J Neurosci*. 2007;27(23):6115–6127.
31. Ligon KL, Alberta JA, Kho AT, et al. The oligodendroglial lineage marker OLIG2 is universally expressed in diffuse gliomas. *J Neuropathol Exp Neurol*. 2004;63(5):499–509.
32. Wang H, Zhang W, et al. Analysis of the activation status of Akt, NFkappaB, and Stat3 in human diffuse gliomas. *Lab Invest*. 2004;84(8):941–951.